AD_____

Award Number DAMD17-98-1-8140

TITLE: Potential Risk of Growth Promoter in Beef for Breast Cancer Growth

PRINCIPAL INVESTIGATOR: Young C. Lin, Ph.D.

CONTRACTING ORGANIZATION: Ohio State University
Columbus, Ohio 43210-1063

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE
Potential Risk of Growth Promoter in Beef for Breast Cancer Growth

5. FUNDING NUMBERS
DAMD17-98-1-8140

6. AUTHOR(S)
Young C. Lin, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Ohio State University
Columbus, Ohio 43210-1063

E-MAIL:

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature Date

Table of Contents

Anabolic agents have been widely used for meat-producing farm animals since such agents were observed to increase weight gain by enhancing protein deposition and improving feed conversion, as well as increasing the muscle-to-fat ratio. Thus, the use of such agents that promote growth, reduce feed costs and improve the quality of the meat produced is an important practice in the meat-producing industry. These anabolic agents have biological activities in common with natural estrogenic, androgenic and progestogenic steroid hormones which have potential effects on protein metabolism. The presence of trace amounts of natural steroid hormones in foodstuffs consumed by humans are easily degraded by the liver, therefore the concern that these natural compounds may pose a health risk to humans is considered negligible.

The project supported by the U.S. Army Medical Research and Materiel Command (USAMRMC) Breast Cancer Research Program is focused on the anabolic compound, zeranol (Ralgro®) which is a non-steroidal agent possessing estrogenic action similar to the natural estrogens. Zeranol has been approved by the Food and Drug Administration (FDA) as a growth promotant for use in the beef, veal, and lamb industries in the U.S. to accelerate growth rate, to improve feed conversion efficiency, and to increase meat-to-fat ratio for the production of a better quality meat to satisfy market demands. The FDA-approved route and dosage of administration is the subcutaneous implantation in the ear of a pellet containing 36 mg of zeranol per beef heifer followed by a second pellet 30 days later. Concerns regarding the potential health risk of zeranol residues in the edible tissues (such as muscle, fat, liver and kidney) of zeranol-implanted beef cattle exist. However, there is no scientific evidence that demonstrates the presence of such a health risk in a convincing manner to the federal regulatory agencies such as the FDA and the United States Department of Agriculture or to the consumers at-large in the United States. It is important to point out that the import of hormone-treated beef from the U.S. to the 15 member countries of the European Union (EU) is banned based on concerns for the potential health risks of the consumption of such beef. Although the U.S. has won an appeal in the World Trade Organization regarding this dispute over hormone-treated beef with the EU, at the present time, the EU still refuses to lift the ban.

The funded research project is designed to examine the effects of zeranol residues present in the serum and extracts of edible tissues collected from beef cattle implanted with zeranol on DNA synthesis in normal human breast cells and human breast cancer cell lines *in vitro*, and subsequently to investigate the *in vivo* effects of the consumption of zeranol residue-containing tissues (in the form of experimentally formulated diets) on the growth of human breast cancer cells in the xenograft-bearing athymic mouse model. The following describes the progress achieved in our laboratory during the past year:

1. Effect of zeranol-containing serum on DNA synthesis in primary cultured normal human breast cells.

Beef cattle were implanted with two zeranol pellets (36 mg zeranol/pellet; sc in the ear) 30 days apart. Serum samples were collected 60 days after implantation of the first pellet. Primary normal human breast cells were cultured in 24-well plates until 75-80% confluent and then treated with fresh medium containing 0, 1, 5 and 25% (v/v) serum from zeranol-treated beef cattle. After 24 hours of treatment, the cells were then pulsed with 5 μCi of ^{3}H-thymidine for 6 hours after which nuclear incorporation of radioactivity was measured. DNA synthesis was expressed as DPM/μg cell protein/6 hours. *Results:* DNA synthesis was significantly elevated in a dose-related fashion by treatment with zeranol-containing serum (25, 70, 180 and 370 DPM/μg cell protein/6 hours by 0, 1, 5, and 25% zeranol-containing serum, respectively). These data imply that circulating levels of zeranol/zeranol residues are capable of enhancing DNA synthesis in cultured normal human breast cells indicating the presence of growth promoting activity in the serum of zeranol-treated cattle. This growth-promoting activity may be the result of the direct action of biologically active zeranol metabolites present in the serum on breast cells or could be due to the ability of zeranol itself or its metabolites to modulate

other not-yet-defined factor(s) within the zeranol-treated cattle which in turn are responsible for the stimulation of breast cell growth. Thus, the question can be raised as to whether the meat from zeranol-implanted beef cattle also contains other biologically active factor(s) or substance(s) that can promote the growth of human breast cancer cells in breast cancer cell-bearing athymic mice consuming experimental diets formulated with meat from zeranol-implanted cattle. The use of this *in vivo* animal model for the funded project will yield experimental data important for addressing the potential effects of the consumption of zeranol-treated meat on human breast cell growth. It is not clear, however, whether our *in vitro* or *in vivo* data can be extrapolated to indicate the presence of a health risk to human females without further experimental evaluation.

2. Biological activity of zeranol extracts from edible meat and fat tissue from beef cattle implanted with zeranol.

Beef cattle were implanted with two zeranol pellets (36 mg zeranol/pellet; sc in the ear) 30 days apart. Muscle (meat) and adipose tissues were collected 60 days after implantation of the first pellet. Primary normal human breast cells were cultured in 24-well plates until 75-80% confluent and then treated with fresh medium containing extracts from the equivalent of 44.4 mg of meat or adipose tissue collected from zeranol-treated beef cattle. After 24 hours of treatment, the cells were then pulsed with 5 μCi of ^{3}H-thymidine for 6 hours after which nuclear incorporation of radioactivity was measured. DNA synthesis was expressed as DPM/μg cell protein/6 hours. *Results:* DNA synthesis was significantly elevated by 27 and 67% after treatment with meat and adipose extracts, respectively. Although the meat and adipose extracts used for treatment were derived from equal amounts of tissue (44.4 mg), the results show a difference in the stimulatory activities of the two types of extracts on human breast cell DNA synthesis. Adipose tissue extract displayed a greater potency than meat extract in stimulating the increase in DNA synthesis. Since the level of HPLC-detectable zeranol in the adipose tissue of zeranol-treated cattle is lower than that in meat (see table below), it is reasonable to assume that this adipose tissue contains zeranol residues/metabolites or other not-yet-defined factor(s) or substance(s) that are more biologically active than those found in meat. This is an important topic deserving of further investigation in the future. If adipose tissue of zeranol-treated cattle is capable of accumulating potent proliferative metabolite(s) or substance(s) through yet unidentified metabolic pathways, then the potential impact of this finding on the health of human consumers of meat from zeranol-treated animals warrants further inquiry in the future.

3. Concentrations of HPLC-detectable zeranol in serum and edible tissues collected from beef cattle implanted with zeranol.

Beef cattle were implanted with two zeranol pellets (36 mg zeranol/pellet; sc in the ear) 30 days apart. Serum and edible tissues were collected 60 days after implantation of the first pellet.

Tissue	HPLC-detectable zeranol (ng/ml serum; ng/mg tissue)
Serum	4.50
Kidney	6.00
Meat	5.50
Liver	3.90

Adipose	3.00

Note: The zeranol levels in serum and edible tissues collected from control cattle were undetectable.

The funded project involves the use of meat from zeranol-implanted cattle to formulate experimental diets to be fed to human breast cancer cell-bearing athymic mice. The level of zeranol present in this meat as determined by HPLC will be considered in the formulation of the experimental diets and to calculate the total amounts of zeranol consumed by the athymic mice during the course of the feeding experiment. As shown in the table above, the level of zeranol in the meat of zeranol-implanted cattle was determined to be 5.50 ng/mg of tissue. Based on this estimation, an athymic mouse that consumes 1 gm of a diet containing 10% zeranol-containing meat will receive about 0.55 μg of HPLC-detected zeranol. We believe that we will be able to use different levels of meat in the experimental diets to examine dose-related promotion of human breast cancer cell growth in the animal model.

4. Effect of zeranol-containing serum on DNA synthesis in estrogen-dependent human breast cancer cell line (MCF-7) and estrogen-independent human breast cancer cell line (MDA-MB-231).

The estrogen receptor (ER)-positive and -negative cell lines MCF-7 and MDA-MB-231, respectively, are routinely cultured in our laboratory for a variety of research projects. Since zeranol is a nonsteroidal agent that exerts estrogenic activity in primary cultured normal human breast cells (see item 1), the dependency of zeranol's effect on ER status for its stimulatory action on DNA synthesis was investigated. The DNA synthesis of MCF-7 and MDA-MB-231 cells was measured by thymidine incorporation assay after 24 hour-treatment with zeranol-containing serum at 0, 1 and 5% (v/v) concentrations in culture medium. *Results:* Treatment with 1 and 5% zeranol-containing serum resulted in statistically significant elevations in DNA synthesis (1500 and 1800 DPM/μg protein/6 hours, respectively) as compared to control (500 DPM/μg protein/6 hours) in MCF-7 cells. The same treatments did not change levels of DNA synthesis in MDA-MB-231 cells. These data suggest that the stimulatory action of zeranol on DNA synthesis in normal human breast cells and in breast cancer cell lines is mediated by an ER-dependent pathway. Since MDA-MB-231 cells do not possess ER, the estrogenic activity present in zeranol-containing serum could not occur. Furthermore, the data show that primary normal human breast cells and the MCF-7 cells show different sensitivities to the proliferative effects of zeranol-containing serum. In primary normal human breast cells, significant stimulation of DNA synthesis required treatment with 5 - 25% zeranol-containing serum, while for MCF-7 cells, 1% zeranol-containing serum was sufficient to induce proliferation. These data provide some intriguing insight into the differential action of a nonsteroidal, estrogenic compound on different human breast cell types. It remains to be determined whether such differential action also occurs under *in vivo* conditions, which is an interesting avenue for further investigation.

5. Mitogenic activity of HPLC-detectable zeranol in meat extracts collected from zeranol-implanted beef cattle.

Beef cattle were implanted with two zeranol pellets (36 mg zeranol/pellet; sc in the ear) 30 days apart. Muscle (meat) tissue was collected 60 days after implantation of the first pellet. Primary normal human breast cells were cultured in 24-well plates until 75-80% confluent and then treated with meat extracts containing 0, 0.34, 1.70 and 8.50 ng zeranol/ml medium. Zeranol levels in the meat extract were determined by HPLC. After 24 hours of treatment, DNA synthesis was measured by ^{3}H-thymidine incorporation assay and expressed as 10^3 DPM/well. *Results:* Treatment with 0, 0.34, 1.70 and 8.50 ng zeranol/ml resulted in 0.05, 1, 4.1 and 10.3 DPM/well (10^3), respectively. The results indicate a dose-related elevation of mitogenic activity with the

lowest dose of 0.34 ng zeranol/ml exerting a significant stimulatory effect. These results indicate the presence of biological activity in stimulating human breast cell proliferation in the extracts of meat collected from zeranol-treated cattle. It is important to point out that the experiment and data described in this item is different from that described in item 2 in which extracts from an equivalent of 44.4mg of meat and adipose tissue were shown to stimulate proliferation of human breast cells. Although the present results correlate specific levels of mitogenic activity with specific doses of HPLC-detected zeranol, we believe that the extracts of meat from zeranol-treated cattle may contain other biologically active components that are not detected by our HPLC system. The identification of these potential compounds would be useful in fully understanding the action of zeranol residue/metabolite-containing food items on estrogen-sensitive tissues. In addition, these data provide a useful guide for estimating the levels of zeranol-containing meat to be included in the formulation of experimental diets for the *in vivo* feeding experiments utilizing the human breast cancer cell-bearing athymic mice.

6. RT-PCR analysis of the effect of zeranol, diethylstilbestrol (DES) and estradiol-17ß (E2) on estrogen-inducible cathepsin D mRNA expression in the ER-positive human breast cancer cell line, MCF-7.

Treatment (ng/ml medium)	Cathepsin D/ß-actin mRNA ratio
Control (0)	0.26
Zeranol (6.45*)	0.55
DES (5.37*)	0.85
E2 (5.45*)	1.10

*: All doses used are equivalent to 20 nM.

Cathepsin D is an estrogen-inducible gene which has been linked to the regulation of normal and cancerous breast cell growth. At an equivalent molar concentration of 20 nM, the potencies of the tested compounds in stimulating cathepsin D mRNA expression are ordered as follows: E2>DES>Zeranol. Since all of the agents used in this experiment were in their pure chemical form, it is difficult to compare these results for zeranol with those generated from the use of meat extracts containing HPLC-detected zeranol. For example, as demonstrated in item 5, HPLC-detected zeranol at a dose of 0.34 ng/ml is capable of stimulating normal human breast cell proliferation. The dose of the pure zeranol used to stimulate cathepsin D expression in MCF-7 cells (20 nM) is 18.97-fold greater. Therefore, the biological activity of meat extracts from zeranol-implanted beef cattle, in terms of estrogen-inducible or -dependent effects, may be more potent than that of zeranol in its pure chemical form. Although evidence derived from *in vitro* experiments may not correlate with results produced by *in vivo* experimentation, the *in vitro* results described here will provide useful information and insight to estimate or calculate the amount of meat from zeranol-implanted cattle needed for the formulation of experimental diets to be used in our *in vivo* feeding experiments utilizing xenograft-bearing athymic mice.

In summary, the progress made in our research project has been useful in providing relevant experimental

data to correctly guide us in the sound design and formulation of experimental diets to be used for the *in vivo* feeding experiments utilizing athymic mice bearing human breast cancer cells. If the results of our *in vitro* experiments can be fully or even partially reproduced in the *in vivo* model, then our expectation for *in vivo* results is that the greater the dosage level of zeranol-containing meat in the diet, the greater the stimulation of human breast cancer cell growth. In other words, we hope to observe a dose-related relationship between levels of zeranol-containing meat consumed and the growth of human breast cancer cells in athymic mice. It is still not clear to us whether the zeranol-containing meat contains other growth-promoting factor(s) or substance(s) in addition to zeranol or its metabolites. In addition, it is also possible that consumption of diets containing meat from zeranol-implanted cattle will cause physiological modulation in the athymic mice that will in turn trigger stimulatory or even inhibitory mechanisms for breast cell growth. Thus, the *in vivo* studies may generate unexpected results.

From our experience, the HPLC measurement of zeranol in the serum and edible tissues of beef cattle implanted with zeranol is an expensive and labor intensive procedure. We hope in the near future, through the use of resources available to us, to produce a specific antibody against biologically active zeranol or its metabolites which will be used to develop an ELISA. If this endeavor is successful, the screening of meat in the market for zeranol and zeranol metabolites can be easily carried out. Information obtained from this approach will be extremely important for policy-making decisions by federal regulatory agencies.